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(71) Applicant: ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(72) Inventor: FARRAH, Theresa, M.; 822 22nd Avenue, Seattle, WA 98122 (US).

(74) Agent: LINGENFELTER, Susan, E.; ZymoGenetics, Inc., 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

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(54) Title: TUMOR NECROSIS FACTOR RECEPTOR ZTNFR-5

(57) Abstract

Novel soluble, secreted tumor necrosis factor receptor (TNRF) polypeptides, polynucleotides encoding the polypeptides, and related compositions and methods are disclosed. The polypeptides comprise four cysteine-rich repeats that are homologous to other tumor necrosis factor receptors, in particular the soluble, secreted tumor necrosis factor receptor osteoprotegerin. The polypeptides may be used for detecting ligands, agonists and antagonists. The polypeptides may also be used in methods that promote cellular maturation and bone cell regulation.

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DESCRIPTION
TUMOR NECROSIS FACTOR RECEPTOR ZTNFR-5

BACKGROUND OF THE INVENTION

Cellular interactions which occur during an immune response are regulated by members of several families of cell surface receptors, including the tumor necrosis factor receptor (TNFR) family. The TNFR family consists of a number of integral membrane glycoprotein receptors many of which, in conjunction with their respective ligands, regulate interactions between different hematopoietic cell lineages (Smith et al., The TNF Receptor Superfamily of Cellular and Viral Proteins: Activation, Costimulation and Death, 76:959-62, 1994; Cosman, Stem Cells 12:440-55, 1994).

The TNF receptor family is composed of a number of type I integral membrane glycoproteins which exhibit sequence homology, particularly with respect to cysteine-rich repeats in their extracellular domains. The TNF receptor family includes p75 NGFR (Johnson et al., Cell 47:545-54, 1989), p55 TNFR-I (Loetscher et al., Cell 61:351-59, 1990), p75 TNFR-II (Schall et al., Cell 61:361-70, 1990), TNFR-RP/TNFR-III (Crowe et al. Science 264:707-10, 1994), CD27 (Camerini et al., J. Immunol. 147:3165-69, 1991), CD30 (Falini et al., Blood 85:1-14, 1995), CD40 (Clark and Lane, Annu. Rev. Immunol. 9:97-127, 1991), 4-1BB (Kwon and Weissman, Proc. Natl. Acad. Sci. USA 86:1963-67, 1989; Schwarz et al., Gene 134:295-298, 1993), OX40 (Malletet al., EMBO J. 9:1063-68, 1990), FAS/APO-1 (Itoh et al., Cell 66:233-43, 1991), DR3 (Chinnaiyan et al., Science 274:990-92, 1996) also known as WSL-1 (Kitson et al., Nature 384:327-75, 1996), DR4 (Pan et al., Science 276:111-13, 1997), osteoprotegerin (OPG) (Simonet et al., Cell 89:309-19, 1997; Human Genome Science, WIPO Publication WO96/28546), CAR1, found in chickens (Brojatsch et al.,

Cell 87:845-55, 1996) plus several viral open reading frames encoding TNFR-related molecules. NGFR, TNFR-I, CD30, CD40, 4-1BB, DR3 and OX40 are mainly restricted to cells of the lymphoid/hematopoietic system. TNRFR-I, TNRFR-II, TNRFR-III and DR4 are found in most human tissues.

Members of the TNF receptor family are characterized by a multi-domain structure comprising an extracellular region, a transmembrane domain, a spacer region between the extracellular ligand-binding region and the transmembrane domain and a cytoplasmic domain, which in several members of this family (TNFR 1, Fas, DR3, DR4, CAR1 and low affinity NGFR) contains a death domain associated with apoptosis. The extracellular ligand-binding region is characterized by the presence of one to six cysteine-rich motifs each containing about six cysteines and approximately 40 amino acids, although variation in the size and number of these motifs occurs among members of this family. The cysteine-rich regions provide the motif for binding to shared structures in the ligands. The highest degree of homology among the TNFR family members is within this extracellular cysteine-rich region. Among human TNFRs the average homology is in the range of 25% to 30%. Between the last cysteine-rich repeat and the transmembrane domain is a small spacer region of between 8 to 70 amino acid residues. Cell surface TNF receptors are anchored in the cell membrane by a transmembrane domain characterized by a sequence of hydrophobic amino acid residues. On the opposite end of the protein from the extracellular ligand-binding region and separated from it by the transmembrane domain is the cytoplasmic domain. The cytoplasmic domains of TNFR family members are small, from 46 to 221 amino acid residues, which suggests possible differences in the signaling mechanisms among family members. In the TNF receptor for example, activation is triggered by the aggregation of cytoplasmic domains of three receptors when their corresponding extracellular domains bind to trimeric ligand.

One member of the TNF receptor family, osteoprotegerin (Simonet et al., *ibid*), is unique in that it is a secreted protein. Soluble forms of other TNF receptors have been described for TNFR-I, TNFR-II, low-affinity NGFR, FAS, CD27, CD30, CD40 and 4-1BB, but these were generated either by cleaving from the cell membrane or secreted by alternatively spliced mRNA. OPG inhibits osteoclast maturation and it is thought that it might serve to regulate bone density by modulating osteoclast differentiation from hematopoietic precursors. OPG provided protection from normal osteoclast remodeling and ovariectomy-associated bone loss.

Ligands for these receptors have been identified, and with one exception (NGF) belong to the TNF ligand family. The members of the TNF ligand family share approximately 20% sequence homology in the extracellular ligand-binding regions, and exist mainly as type II membrane glycoproteins, biologically active as trimeric or multimeric complexes. This group includes TNF, LT-α, LT-β (Browning et al., Cell 72:847-56, 1993), CD27L (Goodwin et al., Cell 73:447-56, 1993), CD30L (Smith et al., Cell 73:1349-60, 1993), CD40L (Armitage et al., Nature 357:80-82, 1992), 4-1BBL (Goodwin et al., Eur. J. Immunol. 23:2631-41, 1993), OX40L (Godfrey et al., J. Exp. Med. 180:757-62, 1994), TRAIL or apo-2 (Wiley et al., Immunity 3:673-82, 1995), TNFγ (Human Genome Sciences, WIPO Publication WO96/14328) and FasL (Cosman, *ibid*,; Lotz et al., J. Leuko. Biol. 60:1-7, 1996). Soluble ligand forms have been identified for TNF, LT-α and FasL. It is not known whether a specific protease cleaves each ligand, releasing it from the membrane, or whether one protease serves the same function for all TNF ligand family members. TACE (TNF-alpha converting enzyme) has been shown to cleave TNF (Moss et al., Nature 385:733-36, 1997; Black et al., Nature 385:729-33, 1997). No other such enzymes are known.

The X-ray crystallographic structures have been resolved for human TNF (Jones et al., Nature 338:225-28,

1989), LT-α (Eck et al., J. Biol. Chem. 267:2119-122, 1992) and the LT-α/TNFR complex (Banner et al., Cell 73:431-45, 1993). This complex features three receptor molecules bound symmetrically to one LT-α trimer. A model of trimeric ligand binding through receptor oligomerization has been proposed to initiate signal transduction pathways. The identification of biological activity of several TNF members has been facilitated through use of monoclonal antibodies specific for the corresponding receptor. These monoclonal antibodies tend to be stimulatory when immobilized and antagonistic in soluble form. This is further evidence that receptor crosslinking is a prerequisite for signal transduction in this receptor family. Importantly, the use of receptor-specific monoclonal antibodies or soluble receptors in the form of multimeric Ig fusion proteins has been useful in determining biological function *in vitro* and *in vivo* for several family members. Soluble receptor-Ig fusion proteins have been used successfully in the cloning of the cell surface ligands corresponding to the CD40, CD30, CD27, 4-1BB and Fas receptors.

In general, the members of the tumor necrosis factor ligand family mediate interactions between different hematopoietic cells, such as T cell/B cell, T cell/monocyte and T cell/T cell interactions. The result of this two-way communication can be stimulatory or inhibitory, depending on the target cell or the activation state. These TNF proteins are involved in regulation of cell proliferation, activation and differentiation, including control of cell survival or death by apoptosis or cytotoxicity. One member of this family, OX-40, is restricted to T cells where it acts as a costimulatory receptor. However, among the TNFR family members there are differences in distribution, kinetics of induction and requirements for induction, which support a defined role for each of the ligands in T cell-mediated immune responses.

The demonstrated *in vitro* and *in vivo* activities of these TNF receptor ligand family members illustrate the enormous clinical potential of, and need for, other TNF receptors, TNF ligands, TNFR agonists, and TNFR antagonists. The present invention addresses this need by providing a novel TNF receptor and related compositions and methods.

SUMMARY OF THE INVENTION

Within one aspect the invention provides an isolated polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2. Within one embodiment the polypeptide comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2. Within another embodiment the polypeptide comprises the region between amino acid residue 1 and amino acid residue 300 of SEQ ID NO:2. Within yet another embodiment the polypeptide further comprises an affinity tag.

Within another aspect the invention provides a fusion protein consisting essentially of a first portion and a second portion joined by a peptide bond, said first portion comprising a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine

residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2; and the second portion comprising another polypeptide. Within one embodiment the second portion is an IgG Fc region.

Within another embodiment is provided a fusion protein comprising a secretory signal sequence having the amino acid sequence of amino acid residues 1-23 of SEQ ID NO:2, wherein said secretory signal sequence is operably linked to an additional polypeptide.

Within yet another embodiment is provided a pharmaceutical composition comprising an isolated polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2; in combination with a pharmaceutically acceptable vehicle.

Within other aspect is provided an isolated polynucleotide encoding an isolated polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2. Within one embodiment the polypeptide comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168,

174 and 193 of SEQ ID NO:2. Within another embodiment the polypeptide comprises the region between amino acid residue 1 and amino acid residue 194 of SEQ ID NO:2. Within a related embodiment the polypeptide further comprises an affinity tag.

Another aspect provided by the invention is an isolated polynucleotide according to claim 11, selected from the group consisting of, a) a polynucleotide having a sequence of nucleotides from nucleotide 252 to nucleotide 764 of SEQ ID NO:1; b) a polynucleotide having a sequence of nucleotides from nucleotide 252 to nucleotide 1082 of SEQ ID NO:1; c) a polynucleotide having a sequence of nucleotides from nucleotide 183 to nucleotide 764 of SEQ ID NO:1; d) a polynucleotide having a sequence of nucleotides from nucleotide 183 to nucleotide 1082 of SEQ ID NO:1; e) a polynucleotide having a sequence of nucleotides from nucleotide 1 to nucleotide 1205 of SEQ ID NO:1; f) nucleotide sequences complementary to a), b), c), d) or e) and g) degenerate nucleotide sequences of a), b), c), d), e) or f).

Within another aspect is an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2; and a transcription terminator. Within one embodiment the DNA segment encodes a polypeptide comprising an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino

acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2. Within another embodiment the DNA segment encodes a polypeptide comprising residues 24-194 of SEQ ID NO:2. Within another embodiment the DNA segment encodes a polypeptide covalently linked amino terminally or carboxy terminally to an affinity tag. Within another embodiment the secretory signal sequence comprises residues 1-23 of SEQ ID NO:2 or SEQ ID NO:44.

Within another aspect is provided a cultured cell into which has been introduced an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2; and a transcription terminator, wherein said cell expresses said polypeptide encoded by said DNA segment.

Also provided is a method of producing a polypeptide comprising: culturing a cell into which has been introduced an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein the polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2; and a transcription terminator; whereby said cell expresses said polypeptide encoded by said DNA segment; and recovering said expressed polypeptide.

Further provided is an antibody that specifically binds to an epitope of a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2.

Also provided is a binding protein that specifically binds to an epitope of a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2.

Another aspect provided is an isolated polynucleotide comprising the sequence of nucleotide 1 to nucleotide 900 of SEQ ID NO:14.

Still further is provided an oligonucleotide probe or primer comprising at least 14 contiguous nucleotides of a polynucleotide of SEQ ID NO:14 or a sequence complementary to SEQ ID NO:14.

These and other aspects of the invention will become evident upon reference to the following detailed description and the attached drawing.

BRIEF DESCRIPTION OF THE DRAWING

The Figure shows a comparison of the deduced amino acid sequence of ZTNFR-5 (SEQ ID NO:2) with the deduced amino acid sequence of osteoprotegerin (SEQ ID NO:3). The predicted cleavage sites for ZTNFR-5 and OPG are marked with double carets, and cysteine-rich repeats #1, #2, #3 and #4 are indicated.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter:

<u>Affinity tag</u>: is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson et al., <u>EMBO J</u>. <u>4</u>:1075, 1985; Nilsson et al., <u>Methods Enzymol</u>. <u>198</u>:3, 1991), glutathione S transferase (Smith and Johnson, <u>Gene</u> <u>67</u>:31, 1988), Glu-Glu affinity tag (Grussenmeyer et al., <u>Proc. Natl. Acad. Sci. USA</u> <u>82</u>:7952-4, 1985), substance P, Flag™ peptide (Hopp et al., <u>Biotechnology</u> <u>6</u>:1204-10, 1988), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford et al., <u>Protein Expression and Purification</u> <u>2</u>: 95-107, 1991. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

<u>Allelic variant</u> : Any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (i.e., no change in the

encoded polypeptide), or may encode polypeptides having altered amino acid sequence. The term "allelic variant" is also used herein to denote a protein encoded by an allelic variant of a gene. Also included are the same protein from the same species which differs from a reference amino acid sequence due to allelic variation. Allelic variation refers to naturally occurring differences among individuals in genes encoding a given protein.

Amino-terminal and carboxyl-terminal: are used herein to denote positions within polypeptides and proteins. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide or protein to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a protein is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete protein.

Complement/anti-complement pair: Denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of $<10^{-9}$ M.

Contig: Denotes a polynucleotide that has a contiguous stretch of identical or complementary sequence to another polynucleotide. Contiguous sequences are said to "overlap" a given stretch of polynucleotide sequence either in their entirety or along a partial stretch of the polynucleotide. For example, representative contigs to the

polynucleotide sequence 5'-ATGGCTTAGCTT-3' are 5'-TAGCTTgagtct-3' and 3'-gtcgacTACCGA-5'.

<u>Complements of polynucleotide molecules</u>: Denotes polynucleotide molecules having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

<u>Degenerate Nucleotide Sequence or Degenerate Sequence</u>: Denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

<u>Expression vector</u>: A DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments may include promoter and terminator sequences, and optionally one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

<u>Isolated</u>: when applied to a polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will

be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316:774-78, 1985).

Isolated polypeptide or protein: is a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

Operably linked: As applied to nucleotide segments, the term "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator.

Ortholog: Denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

Paralogs: Are distinct but structurally related proteins made by an organism. Paralogs are believed to arise through gene duplication. For example, α-globin, β-globin, and myoglobin are paralogs of each other.

Polynucleotide: denotes a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the

context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

<u>Polypeptide</u>: Is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

<u>Promoter</u>: Denotes a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

<u>Protein</u>: is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

<u>Receptor</u>: A cell-associated protein, or a polypeptide subunit of such protein, that binds to a bioactive molecule (the "ligand") and mediates the effect of the ligand on the cell. Binding of ligand to receptor results in a change in the receptor (and, in some cases, receptor multimerization, i.e., association of identical or different receptor subunits) that causes interactions

between the effector domain(s) of the receptor and other molecule(s) in the cell. These interactions in turn lead to alterations in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, cell proliferation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. ZTNFR-5 has characteristics of TNF receptors, as discussed in more detail below.

Secretory signal sequence: A DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

Soluble receptor: A receptor polypeptide that is not bound to a cell membrane. Soluble receptors are most commonly ligand-binding receptor polypeptides that lack transmembrane and cytoplasmic domains. Soluble receptors can comprise additional amino acid residues, such as affinity tags that provide for purification of the polypeptide or provide sites for attachment of the polypeptide to a substrate. Many cell-surface receptors have naturally occurring, soluble counterparts that are produced by proteolysis or translated from alternatively spliced mRNAs. Receptor polypeptides are said to be substantially free of transmembrane and intracellular polypeptide segments when they lack sufficient portions of these segments to provide membrane anchoring or signal transduction, respectively.

Molecular weights and lengths of polymers determined by imprecise analytical methods (e.g., gel electrophoresis) will be understood to be approximate values. When such a value is expressed as "about" X or

"approximately" X, the stated value of X will be understood to be accurate to ±10%.

All references cited herein are incorporated by reference in their entirety.

The present invention is based in part upon the discovery of a novel 1205 bp DNA sequence (SEQ ID NO:1) and corresponding polypeptide sequence (SEQ ID NO:2) which have homology to members of the tumor necrosis factor receptor family. The receptor has been designated ZTNFR-5. Novel ZTNFR-5 receptor-encoding polynucleotides and polypeptides of the present invention were initially identified by querying an EST (Expressed Sequence Tag) database for sequences homologous to conserved motifs within the TNF receptor family. Based on this search a contig of 16 ESTs was constructed. A comparison of the ZTNFR-5 deduced amino acid sequence (as represented in SEQ ID NO:2) with the deduced amino acid sequence of human osteoprotegerin (OPG) (SEQ ID NO:3) is shown in the Figure.

Structurally, the TNF receptor family is characterized by an extracellular portion composed of several modules called, historically, "cysteine-rich pseudo-repeats". A prototypical family member has four of these pseudo-repeats, each about 29-43 residues long, one right after the other. A typical pseudo-repeat has 6 cysteine residues. They are called pseudo-repeats because, although they appear to originate from a common ancestral module, they do not repeat exactly: pseudo-repeats #1, #2, #3 and #4 have characteristic sequence features which distinguish them from one another. The crystal structure of the p55 TNF receptor revealed that each pseudo-repeat corresponds to one folding domain, and that all four pseudo-repeats fold into the same tertiary structure, held together internally by disulfide bonds.

Sequence motifs for the four sequence motifs are shown below. Within in the motif, X represents an amino acid residue, the numbers contained within curly brackets are multipliers for the preceding residue and residues in

brackets are optional and alternative sequences are indicated with asterisks.

Pseudo repeat #1 motif (SEQ ID NO:28)

X-C-X{10-14}-C-C-X-X-C-X{5-9}-C-X{6-8}-C-X

Pseudo-repeat #1 of ZTNFR-5 (amino acid residues 49-71 of SEQ ID NO:2) is missing the first two cysteines of this motif.

Pseudo-repeat #2 motif (SEQ ID NO:29)

X-C-X{13-15}-C-X-X-C-X{2-3}-C-X{8-11}-C-X{7}-C

Pseudo-repeat #2 of ZTNFR-5 (amino acid residues 72-113 of SEQ ID NO:2) match this motif.

Pseudo-repeat #3 motif (SEQ ID NO:30)

```
X-C-X{5-6}-X-X{4-9}-C-X-X-C-X{2-7}-C-X{8-9}-C-X{7}-C-(X)
     *                                 ************
     C                               X{10-16} (SEQ ID NO:31)
```

Pseudo-repeat #3 of ZTNFR-5 (amino acid residues 114-151 SEQ ID NO:2) is encompassed within the alternative sequences.

Pseudo-repeat #4 motif (SEQ ID NO:32)

```
X-C-X{10-14}-C-X-X-C-X-X-C-X{4-10}-C-X{3-7}-C-X
                 *              *
                 X              X (SEQ ID
NO:33)
```

Pseudo-repeat #4 of ZTNFR-5 (amino acid residues 151-194 of SEQ ID NO:2) is encompassed within the alternative sequences.

Sequence analysis of a deduced amino acid sequence of ZTNFR-5, as represented in SEQ ID NO:2 indicates the presence of 23 amino acid residues of a signal peptide (residues 1-23 of SEQ ID NO:2); and a mature protein (residues 24-300) containing four extracellular, cysteine-rich pseudo-repeats (residues 49-71, 72-113, 114-151 and 152-194 of SEQ ID NO:2). A typical pseudo-repeat has six cysteines. Pseudo-repeat #1 has conserved cysteine residues as amino acid residues 49, 52, 62 and 70 of SEQ ID

NO:2. Pseudo-repeat #2 has conserved cysteine residues at amino acid residues 73, 88, 91, 95, 105 and 113 of SEQ ID NO:2. Pseudo-repeat has conserved cysteine residues at amino acid residues 115, 126, 132 and 150 of SEQ ID NO:2. Pseudo-repeat #4 has conserved cysteine residues at amino acid residues 153, 168, 174 and 193 of SEQ ID NO:2. Those skilled in the art will recognize that these domain boundaries are approximate, and are based on alignments with known proteins and predictions of protein folding. These features indicate that the receptor encoded by the DNA sequence of SEQ ID NO:1 is a member of the TNF receptor family.

The four cysteine-rich domains of the extracellular ligand binding region of ZTNFR-5 are similar to several other members of the TNF receptor family, in particular, OPA and TNFR-2. ZTNFR-5 shares an overall 31% amino acid identity with OPG. The greatest homology, 42%, is within the four cysteine-rich repeats. Like OPG, ZTNFR-5 is a soluble, secreted TNF receptor, however it may also exist, as do other TNFRs, as a membrane bound receptor having a transmembrane domain and a cytoplasmic domain.

Northern blot analysis, using a 360 bp probe to the predicted N-terminus of the deduced amino acid sequence encoded by the contig, resulted in a 1.2bp transcript which was strongly expressed in lung, spinal cord, stomach, lymph node, spleen, colon and trachea. Less intense signals were present in kidney, thymus, heart, pancreas, prostate, small intestine, placenta, thyroid, and bone marrow tissue. In the case of lung, a 2.4kb transcript was also detected. Broad tissue distribution is not unknown in the TNF receptor family. Several members, TNRFR-I, TNRFR-II, TNRFR-III and DR4, are found in most human tissues. OPG was detected in lung, heart, kidney, placenta, and to a lesser degree, in hematopoietic and immune organs (Simmonet et al., <u>ibid</u>.). Several ESTs which made up the ZTNFR-5 contig were from tumor libraries, prostate, colon and breast. Expression in tumor cells is consistent with

other members of the TNFR family that are associated with growth regulation, differentiation and tumorigenesis.

The present invention also provides polynucleotide molecules, including DNA and RNA molecules, that encode the ZTNFR-5 polypeptides disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. SEQ ID NO:14 is a degenerate DNA sequence that encompasses all DNAs that encode the ZTNFR-5 polypeptide of SEQ ID NO:2. Those skilled in the art will recognize that the degenerate sequence of SEQ ID NO:14 also provides all RNA sequences encoding SEQ ID NO:2 by substituting U (uracil) for T (thymine). Thus, ZTNFR-5 polypeptide-encoding polynucleotides comprising nucleotide 1 to nucleotide 900 of SEQ ID NO:14 and their RNA equivalents are contemplated by the present invention. Table 1 sets forth the one-letter codes used within SEQ ID NO:14 to denote degenerate nucleotide positions. "Resolutions" are the nucleotides denoted by a code letter. "Complement" indicates the code for the complementary nucleotide(s). For example, the code Y denotes either C (cytosine) or T, and its complement R denotes A (adenine) or G (guanine), A being complementary to T, and G being complementary to C.

TABLE 1

Nucleotide	Resolution	Complement	Resolution
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A|G	Y	C|T
Y	C|T	R	A|G
M	A|C	K	G|T
K	G|T	M	A|C
S	C|G	S	C|G
W	A|T	W	A|T
H	A|C|T	D	A|G|T
B	C|G|T	V	A|C|G
V	A|C|G	B	C|G|T
D	A|G|T	H	A|C|T
N	A|C|G|T	N	A|C|G|T

The degenerate codons used in SEQ ID NO:14, encompassing all possible codons for a given amino acid, are set forth in Table 2.

TABLE 2

Amino Acid	One Letter Code	Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn|Asp	B		RAY
Glu|Gln	Z		SAR
Any	X		NNN

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO:2. Variant sequences can be readily tested for functionality as described herein.

One of ordinary skill in the art will also appreciate that different species can exhibit "preferential codon usage." In general, see, Grantham, et al., Nuc. Acids Res. 8:1893-912, 1980; Haas, et al. Curr. Biol. 6:315-24, 1996; Wain-Hobson, et al., Gene 13:355-64, 1981; Grosjean and Fiers, Gene 18:199-209, 1982; Holm, Nuc. Acids Res. 14:3075-87, 1986; Ikemura, J. Mol. Biol. 158:573-97, 1982. As used herein, the term "preferential codon usage" or "preferential codons" is a term of art referring to protein translation codons that are most frequently used in cells of a certain species, thus favoring one or a few representatives of the possible codons encoding each amino acid (See Table 2). For example, the amino acid threonine (Thr) may be encoded by ACA, ACC, ACG, or ACT, but in mammalian cells ACC is the most commonly used codon; in other species, for example, insect cells, yeast, viruses or bacteria, different Thr codons may be preferential. Preferential codons for a particular species can be introduced into the polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferential codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within

a particular cell type or species. Therefore, the degenerate codon sequence disclosed in SEQ ID NO:17 serves as a template for optimizing expression of polynucleotides in various cell types and species commonly used in the art and disclosed herein. Sequences containing preferential codons can be tested and optimized for expression in various species, and tested for functionality as disclosed herein.

The highly conserved amino acids in the [a significant domain, region or motif] of ZTNFR-5 can be used as a tool to identify new family members. For instance, reverse transcription-polymerase chain reaction (RT-PCR) can be used to amplify sequences encoding the extracellular ligand-binding domain, described above, from RNA obtained from a variety of tissue sources or cell lines. In particular, highly degenerate primers designed from the ZTNFR-5 sequences are useful for this purpose.

Within preferred embodiments of the invention, isolated polynucleotides will hybridize to similar sized regions of SEQ ID NO:1, or to a sequence complementary thereto, under stringent conditions. In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typical stringent conditions are those in which the salt concentration is up to about 0.03 M at pH 7 and the temperature is at least about 60°C.

As previously noted, the isolated polynucleotides of the present invention include DNA and RNA. Methods for isolating DNA and RNA are well known in the art. It is generally preferred to isolate RNA from lung or lymphoid tissue, although DNA can also be prepared using RNA from other tissues or isolated as genomic DNA. Total RNA can be prepared using guanidine HCl extraction followed by isolation by centrifugation in a CsCl gradient (Chirgwin et

al., Biochemistry 18:52-94, 1979). Poly $(A)^+$ RNA is prepared from total RNA using the method of Aviv and Leder (Proc. Natl. Acad. Sci. USA 69:1408-12, 1972). Complementary DNA (cDNA) is prepared from poly$(A)^+$ RNA using known methods. Polynucleotides encoding ZTNFR-5 polypeptides are then identified and isolated by, for example, hybridization or PCR.

Those skilled in the art will recognize that the sequence disclosed in SEQ ID NO:1 represents a single allele of the human gene, and that allelic variation and alternative splicing is expected to occur. Allelic variants of the DNA sequence shown in SEQ ID NO:1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NO:2. cDNAs generated from alternatively spliced mRNAs, which retain the properties of the ZTNFR-5 polypeptide are included within the scope of the present invention, as are polypeptides encoded by such cDNAs and mRNAs. Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

The present invention further provides counterpart receptors and polynucleotides from other species (orthologs). These species include, but are not limited to mammalian, avian, amphibian, reptile, fish, insect and other vertebrate and invertebrate species. Of particular interest are ZTNFR-5 receptors from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primate receptors. Orthologs of the human ZTNFR-5 receptor can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses the receptor. Suitable sources of mRNA can be identified by

probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A receptor-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequence. A cDNA can also be cloned using PCR, using primers designed from the sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to the receptor. Similar techniques can also be applied to the isolation of genomic clones.

The present invention also provides isolated receptor polypeptides that are substantially homologous to the receptor polypeptide of SEQ ID NO:2 and its orthologs. It is preferred to provide the proteins or polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. The term "substantially homologous" is used herein to denote proteins or polypeptides having 50%, preferably 60%, more preferably at least 80%, sequence identity to the sequence shown in SEQ ID NO:2 or its orthologs. Such proteins or polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO:2 or its orthologs. Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48: 603-16, 1986 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-19, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (ibid.) as shown in Table 3 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{\text{[length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences]}} \times 100$$

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Table 3

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Substantially homologous proteins and polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 4) and other substitutions that do not significantly affect the folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or an affinity tag. Polypeptides comprising affinity tags can further comprise a proteolytic cleavage site between the ZTNFR-5 polypeptide and the affinity tag. Preferred such sites include thrombin cleavage sites and factor Xa cleavage sites.

Table 4

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins.

For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., J. Am. Chem. Soc. 113:2722, 1991; Ellman et al., Methods Enzymol. 202:301, 1991; Chung et al., Science 259:806-9, 1993; and Chung et al., Proc. Natl. Acad. Sci. USA 90:10145-9, 1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., J. Biol. Chem. 271:19991-8, 1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide et al., Biochem. 33:7470-6, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, Protein Sci. 2:395-403, 1993).

A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for ZTNFR-5 amino acid residues.

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244: 1081-5, 1989; Bass et al., Proc. Natl. Acad. Sci. USA 88:4498-502, 1991). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity as disclosed below to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton et al., J. Biol. Chem. 271:4699-708, 1996. Sites of ligand-receptor interaction can also be determined by physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., Science 255:306-12, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992. The identities of essential amino acids can also be inferred from analysis of homologies with related tumor necrosis factor receptors such as osteoprotegerin.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-7, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-6, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-7, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and

region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

Variants of the disclosed ZTNFR-5 DNA and polypeptide sequences can be generated through DNA shuffling as disclosed by Stemmer, Nature 370:389-91, 1994, Stemmer, Proc. Natl. Acad. Sci. USA 91:10747-51, 1994 and WIPO Publication WO 97/20078. Briefly, variant DNAs are generated by *in vitro* homologous recombination by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent DNAs, such as allelic variants or DNAs from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed above can be combined with high-throughput screening methods to detect activity of cloned, mutagenized receptors in host cells. Mutagenized DNA molecules that encode active receptors or portions thereof (e.g., ligand-binding fragments) can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

Using the methods discussed above, one of ordinary skill in the art can identify and/or prepare a variety of polypeptides that are substantially homologous to residues 24-194 of SEQ ID NO: 2 or allelic variants thereof and retain the ligand-binding properties of the wild-type protein. Such polypeptides may include additional amino acids from affinity tags and the like.

Such polypeptides may also include additional polypeptide segments as generally disclosed above.

The receptor polypeptides of the present invention, including full-length receptor polypeptides, receptor fragments (e.g., soluble ligand-binding fragments), and fusion polypeptides, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; and Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987.

In general, a DNA sequence encoding a ZTNFR-5 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a ZTNFR-5 polypeptide into the secretory pathway of a host cell, a secretory signal

sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of the ZTNFR-5 polypeptide, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*. The secretory signal sequence is joined to the ZTNFR-5 DNA sequence in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Cultured mammalian cells are suitable hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-45, 1982), DEAE-dextran mediated transfection (Ausubel et al., ibid.), and liposome-mediated transfection (Hawley-Nelson et al., Focus 15:73, 1993; Ciccarone et al., Focus 15:80, 1993). The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977) and Chinese hamster ovary (e.g., CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the

American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978 and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g., hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used. Alternative markers that introduce an altered phenotype, such as green fluorescent protein, or cell surface proteins such as CD4, CD8, Class I MHC, placental alkaline phosphatase may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

Other higher eukaryotic cells can also be used as hosts, including plant cells, insect cells and avian

cells. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., J. Biosci. (Bangalore) 11:47-58, 1987. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino et al., U.S. Patent No. 5,162,222 and WIPO publication WO 94/06463. Insect cells can be infected with recombinant baculovirus, commonly derived from *Autographa californica nuclear polyhedrosis virus* (AcNPV). See, King and Possee, The Baculovirus Expression System: A Laboratory Guide, London, Chapman & Hall; O'Reilly et al., Baculovirus Expression Vectors: A Laboratory Manual, New York, Oxford University Press., 1994; and Richardson, Ed., Baculovirus Expression Protocols. Methods in Molecular Biology, Totowa, NJ, Humana Press, 1995. A second method of making recombinant ZTNFR-5 baculovirus utilizes a transposon-based system described by Luckow (Luckow, et al., J Virol 67:4566-79, 1993). This system, which utilizes transfer vectors, is sold in the Bac-to-Bac™ kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, pFastBac1™ (Life Technologies) containing a Tn7 transposon to move the DNA encoding the ZTNFR-5 polypeptide into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." See, Hill-Perkins and Possee, J. Gen. Virol. 71:971-6, 1990; Bonning, et al., J. Gen. Virol. 75:1551-6, 1994; and, Chazenbalk, and Rapoport, J. Biol. Chem. 270:1543-9, 1995. In addition, transfer vectors can include an in-frame fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed ZTNFR-5 polypeptide, for example, a Glu-Glu epitope tag (Grussenmeyer et al., Proc. Natl. Acad. Sci. 82:7952-4, 1985). Using a technique known in the art, a transfer vector containing ZTNFR-5 is transformed into *E. coli*, and screened for bacmids which contain an interrupted lacZ gene indicative of recombinant

baculovirus. The bacmid DNA containing the recombinant baculovirus genome is isolated, using common techniques, and used to transfect *Spodoptera frugiperda* cells, e.g. Sf9 cells. Recombinant virus that expresses ZTNFR-5 is subsequently produced. Recombinant viral stocks are made by methods commonly used the art.

The recombinant virus is used to infect host cells, typically a cell line derived from the fall armyworm, *Spodoptera frugiperda*. See, in general, Glick and Pasternak, Molecular Biotechnology: Principles and Applications of Recombinant DNA, ASM Press, Washington, D.C., 1994. Another suitable cell line is the High FiveO™ cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent #5,300,435). Commercially available serum-free media are used to grow and maintain the cells. Suitable media are Sf900 II™ (Life Technologies) or ESF 921™ (Expression Systems) for the Sf9 cells; and Ex-cellO405™ (JRH Biosciences, Lenexa, KS) or Express FiveO™ (Life Technologies) for the *T. ni* cells. The cells are grown up from an inoculation density of approximately 2-5 x 10^5 cells to a density of 1-2 x 10^6 cells at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3. Procedures used are generally described in available laboratory manuals (King and Possee, ibid.; O'Reilly, et al., ibid.; Richardson, ibid.). Subsequent purification of the ZTNFR-5 polypeptide from the supernatant can be achieved using methods described herein.

Fungal cells, including yeast cells, can also be used within the present invention. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides

therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces fragilis, Ustilago maydis, Pichia pastoris, Pichia methanolica, Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., J. Gen. Microbiol. 132:3459-65, 1986 and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

The use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed in WIPO Publications WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565. DNA molecules for use in transforming *P.*

methanolica will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For polypeptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (*AUG1* or *AUG2*). Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. A preferred selectable marker for use in *Pichia methanolica* is a *P. methanolica ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), which allows *ade2* host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. It is preferred to transform *P. methanolica* cells by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (τ) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus* and other genera are also useful host cells within the present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art (see, e.g., Sambrook et al., ibid.). When expressing

a ZTNFR-5 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell. *P. methanolica* cells are cultured in a medium comprising adequate sources of carbon, nitrogen and trace nutrients at a temperature of about 25°C to 35°C. Liquid cultures are provided with sufficient aeration by conventional means, such as shaking of small flasks or sparging of fermentors. A preferred

culture medium for *P. methanolica* is YEPD (2% D-glucose, 2% Bacto™ Peptone (Difco Laboratories, Detroit, MI), 1% Bacto™ yeast extract (Difco Laboratories), 0.004% adenine and 0.006% L-leucine).

Expressed recombinant ZTNFR-5 polypeptides (or chimeric ZTNFR-5 polypeptides) can be purified using fractionation and/or conventional purification methods and media. Ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable anion exchange media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred, with DEAE Fast-Flow Sepharose (Pharmacia, Piscataway, NJ) being particularly preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and

are available from commercial suppliers. Methods for binding receptor polypeptides to support media are well known in the art. Selection of a particular method is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988.

The polypeptides of the present invention can be isolated by exploitation of their physical properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins including those comprising polyhistidine tags. Briefly, a gel is first charged with divalent metal ions to form a chelate (Sulkowski, Trends in Biochem. 3:1-7, 1985). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography (Methods in Enzymol., Vol. 182, "Guide to Protein Purification", M. Deutscher, (ed.), Acad. Press, San Diego, 1990, pp.529-39). Within additional embodiments of the invention, a fusion of the polypeptide of interest and an affinity tag (e.g., maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification.

Protein refolding (and optionally reoxidation) procedures may be advantageously used. It is preferred to purify the protein to >80% purity, more preferably to >90% purity, even more preferably >95%, and particularly preferred is a pharmaceutically pure state, that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. Preferably, a purified protein is substantially free of

other proteins, particularly other proteins of animal origin.

ZTNFR-5 polypeptides or fragments thereof may also be prepared through chemical synthesis. ZTNFR-5 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not include an initial methionine amino acid residue.

The invention also provides soluble ZTNFR-5 receptors used to form fusion proteins with human Ig, to form His-tagged proteins, or FLAG™-tagged proteins. Soluble ZTNFR-5 or ZTNFR-5-Ig fusion proteins are used, for example, to identify the ZTNFR-5 ligands, including the natural ligand, as well as agonists and antagonists of the natural ligand. Using labeled soluble ZTNFR-5, cells expressing the ligand are identified by fluorescence immunocytometry or immunohistochemistry. The soluble fusion proteins or soluble Ig fusion proteins are useful in studying the distribution of the ligand on tissues or specific cell lineages, and to provide insight into receptor/ligand biology.

Immunoglobulin-ZTNFR-5 polypeptide fusions can be expressed in genetically engineered cells to produce a variety of multimeric ZTNFR-5 analogs. Auxiliary domains can be fused to ZTNFR-5 polypeptides to target them to specific cells, tissues, or macromolecules. For example, a ZTNFR-5 polypeptide or protein could be targeted to a predetermined cell type by fusing a ZTNFR-5 polypeptide to a ligand that specifically binds to a receptor on the surface of the target cell. In this way, polypeptides and proteins can be targeted for therapeutic or diagnostic purposes. A ZTNFR-5 polypeptide can be fused to two or more moieties, such as an affinity tag for purification and a targeting domain. Polypeptide fusions can also comprise one or more cleavage sites, particularly between domains. See, Tuan et al., Connective Tissue Research 34:1-9, 1996. Construction of a soluble ZTNFR-5-IgG

fusion protein is described in more detail it the Example section.

Polypeptides containing an amino acid sequence associated with ligand-binding (such as residues 24-194 of SEQ ID NO:2) can be used for purification of ligand. The receptor polypeptide is immobilized on a solid support, such as beads of agarose, cross-linked agarose, glass, cellulosic resins, silica-based resins, polystyrene, cross-linked polyacrylamide, or like materials that are stable under the conditions of use. Methods for linking polypeptides to solid supports are known in the art, and include amine chemistry, cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, and hydrazide activation. The resulting media will generally be configured in the form of a column, and fluids containing ligand are passed through the column one or more times to allow ligand to bind to the receptor polypeptide. The ligand is then eluted using changes in salt concentration, chaotropic agents ($MnCl_2$), or pH to disrupt ligand-receptor binding.

To direct the export of the receptor domain from the host cell, the receptor DNA is linked to a second DNA segment encoding a secretory peptide, such as a t-PA secretory peptide. To facilitate purification of the secreted receptor domain, an N- or C-terminal extension, such as a poly-histidine tag, substance P, FLAG™ peptide (Hopp et al., <u>Biotechnology</u> <u>6</u>:1204-10, 1988; available from Eastman Kodak Co., New Haven, CT) or another polypeptide or protein for which an antibody or other specific binding agent is available, can be fused to the receptor polypeptide.

In an alternative approach, a soluble ZTNFR-5 receptor extracellular ligand-binding region can be expressed as a fusion with immunoglobulin heavy chain constant regions, typically an F_C fragment, which contains two constant region domains and a hinge region, but lacks

the variable region. Such fusions are typically secreted as multimeric molecules, wherein the Fc portions are disulfide bonded to each other and two receptor polypeptides are arrayed in close proximity to each other. Fusions of this type can be used to affinity purify the cognate ligand from solution, as an *in vitro* assay tool, to block signals *in vitro* by specifically titrating out ligand, and as antagonists *in vivo* by administering them to block ligand stimulation. To purify ligand, a ZTNFR-5-Ig fusion protein (chimera) is added to a sample containing the ligand under conditions that facilitate receptor-ligand binding (typically near-physiological temperature, pH, and ionic strength). The chimera-ligand complex is then separated by the mixture using protein A, which is immobilized on a solid support (e.g., insoluble resin beads). The ligand is then eluted using conventional chemical techniques, such as with a salt or pH gradient. In the alternative, the chimera itself can be bound to a solid support, with binding and elution carried out as above. For use in assays, the chimeras are bound to a support via the F_C region and used in an ELISA format.

Cells expressing functional receptor are used within screening assays. A variety of suitable assays are known in the art. These assays are based on the detection of a biological response in a target cell. An increase in metabolism above a control value indicates a test compound that modulates ZTNFR-5 mediated metabolism. One such assay is a cell proliferation assay. Cells are cultured in the presence or absence of a test compound, and cell proliferation is detected by, for example, measuring incorporation of tritiated thymidine or by colorimetric assay based on the metabolic breakdown of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Mosman, J. Immunol. Meth. 65: 55-63, 1983). An alternative assay format uses cells that are further

engineered to express a reporter gene. The reporter gene is linked to a promoter element that is responsive to the receptor-linked pathway, and the assay detects activation of transcription of the reporter gene. Numerous reporter genes that are easily assayed for in cell extracts are known in the art, for example, the *E. coli lacZ*, chloroamphenicol acetyl transferase (CAT) and serum response element (SRE) (see, e.g., Shaw et al., Cell 56:563-72, 1989). A preferred such reporter gene is a luciferase gene (de Wet et al., Mol. Cell. Biol. 7:725, 1987). Expression of the luciferase gene is detected by luminescence using methods known in the art (e.g., Baumgartner et al., J. Biol. Chem. 269:29094-101, 1994; Schenborn and Goiffin, Promega Notes 41:11, 1993). Luciferase activity assay kits are commercially available from, for example, Promega Corp., Madison, WI. Target cell lines of this type can be used to screen libraries of chemicals, cell-conditioned culture media, fungal broths, soil samples, water samples, and the like. For example, a bank of cell-conditioned media samples can be assayed on a target cell to identify cells that produce ligand. Positive cells are then used to produce a cDNA library in a mammalian expression vector, which is divided into pools, transfected into host cells, and expressed. Media samples from the transfected cells are then assayed, with subsequent division of pools, re-transfection, subculturing, and re-assay of positive cells to isolate a cloned cDNA encoding the ligand.

An assay system that uses a ligand-binding receptor (or an antibody, one member of a complement/anti-complement pair) or a binding fragment thereof, and a commercially available biosensor instrument (BIAcore™, Pharmacia Biosensor, Piscataway, NJ) may also may be advantageously employed. Such receptor, antibody, member of a complement/anti-complement pair or fragment is immobilized onto the surface of a receptor chip. Use of

this instrument is disclosed by Karlsson, J. Immunol. Methods 145:229-40, 1991 and Cunningham and Wells, J. Mol. Biol. 234:554-63, 1993. A receptor, antibody, member or fragment is covalently attached, using amine or sulfhydryl chemistry, to dextran fibers that are attached to gold film within the flow cell. A test sample is passed through the cell. If a ligand, epitope, or opposite member of the complement/anti-complement pair is present in the sample, it will bind to the immobilized receptor, antibody or member, respectively, causing a change in the refractive index of the medium, which is detected as a change in surface plasmon resonance of the gold film. This system allows the determination of on- and off-rates, from which binding affinity can be calculated, and assessment of stoichiometry of binding. Ligand-binding receptor polypeptides can also be used within other assay systems known in the art. Such systems include Scatchard analysis for determination of binding affinity (see, Scatchard, Ann. NY Acad. Sci. 51: 660-72, 1949) and calorimetric assays (Cunningham et al., Science 253:545-48, 1991; Cunningham et al., Science 245:821-25, 1991).

The soluble ZTNFR-5 is useful in studying the distribution of ligands on tissues or specific cell lineages, and to provide insight into receptor/ligand biology. Application may also be made of the specificity of TNF receptors for their ligands as a mechanism by which to destroy ligand-bearing target cells. For example, toxic compounds may be coupled to ZTNFR-5 receptors, in particular to soluble receptors. Examples of toxic compounds would include radiopharmaceuticals that inactivate target cells; chemotherapeutic agents such as doxorubicin, daunorubicin, methotrexate, and cytoxan; toxins, such as ricin, diphtheria, Pseudomonas exotoxin A and abrin; and antibodies to cytotoxic T-cell surface molecules.

ZTNFR-5 polynucleotides and/or polypeptides may be useful for regulating the maturation of TNF ligand-bearing cells, such as T cells, B cells, lymphocytes, peripheral blood mononuclear cells, polymorphonuclear leukocytes, fibroblasts and hematopoietic cells. ZTNFR-5 polypeptides will also find use in mediating metabolic or physiological processes *in vivo*. Proliferation and differentiation can be measured *in vitro* using cultured cells. Bioassays and ELISAs are available to measure cellular response to ZTNFR-5, in particular are those which measure changes in cytokine production as a measure of cellular response (see for example, Current Protocols in Immunology ed. John E. Coligan et al., NIH, 1996). Assays to measure other cellular responses, including antibody isotype, monocyte activation, NK cell formation, antigen presenting cell function, apoptosis are known in the art.

In vitro and *in vivo* response to soluble ZTNFR-5 can also be measured using cultured cells or by administering molecules of the claimed invention to the appropriate animal model. For instance, soluble ZTNFR-5 transfected expression host cells may be embedded in an alginate environment and injected (implanted) into recipient animals. Alginate-poly-L-lysine microencapsulation, permselective membrane encapsulation and diffusion chambers have been described as a means to entrap transfected mammalian cells or primary mammalian cells. These types of non-immunogenic "encapsulations" or microenvironments permit the transfer of nutrients into the microenvironment, and also permit the diffusion of proteins and other macromolecules secreted or released by the captured cells across the environmental barrier to the recipient animal. Most importantly, the capsules or microenvironments mask and shield the foreign, embedded cells from the recipient animal's immune response. Such microenvironments can extend the life of the injected

cells from a few hours or days (naked cells) to several weeks (embedded cells).

Alginate threads provide a simple and quick means for generating embedded cells. The materials needed to generate the alginate threads are readily available and relatively inexpensive. Once made, the alginate threads are relatively strong and durable, both *in vitro* and, based on data obtained using the threads, *in vivo*. The alginate threads are easily manipulable and the methodology is scalable for preparation of numerous threads. In an exemplary procedure, 3% alginate is prepared in sterile H_2O, and sterile filtered. Just prior to preparation of alginate threads, the alginate solution is again filtered. An approximately 50% cell suspension (containing about 5×10^5 to about 5×10^7 cells/ml) is mixed with the 3% alginate solution. One ml of the alginate/cell suspension is extruded into a 100 mM sterile filtered $CaCl_2$ solution over a time period of ~15 min, forming a "thread". The extruded thread is then transferred into a solution of 50 mM $CaCl_2$, and then into a solution of 25 mM $CaCl_2$. The thread is then rinsed with deionized water before coating the thread by incubating in a 0.01% solution of poly-L-lysine. Finally, the thread is rinsed with Lactated Ringer's Solution and drawn from solution into a syringe barrel (without needle attached). A large bore needle is then attached to the syringe, and the thread is intraperitoneally injected into a recipient in a minimal volume of the Lactated Ringer's Solution.

An alternative *in vivo* approach for assaying proteins of the present invention involves viral delivery systems. Exemplary viruses for this purpose include adenovirus, herpesvirus, vaccinia virus and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acid (for a review, see Becker et al., Meth. Cell Biol. 43:161-89, 1994; and

Douglas and Curiel, Science & Medicine 4:44-53, 1997). The adenovirus system offers several advantages: adenovirus can (i) accommodate relatively large DNA inserts; (ii) be grown to high-titer; (iii) infect a broad range of mammalian cell types; and (iv) be used with a large number of available vectors containing different promoters. Also, because adenoviruses are stable in the bloodstream, they can be administered by intravenous injection.

By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts may be incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene has been deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell (the human 293 cell line is exemplary). When intravenously administered to intact animals, adenovirus primarily targets the liver. If the adenoviral delivery system has an E1 gene deletion, the virus cannot replicate in the host cells. However, the host's tissue (e.g., liver) will express and process (and, if a signal sequence is present, secrete) the heterologous protein. Secreted proteins will enter the circulation in the highly vascularized liver, and effects on the infected animal can be determined.

The adenovirus system can also be used for protein production *in vitro*. By culturing adenovirus-infected non-293 cells under conditions where the cells are not rapidly dividing, the cells can produce proteins for extended periods of time. For instance, BHK cells are grown to confluence in cell factories, then exposed to the adenoviral vector encoding the secreted protein of interest. The cells are then grown under serum-free conditions, which allows infected cells to survive for several weeks without significant cell division.

Alternatively, adenovirus vector infected 293S cells can be grown in suspension culture at relatively high cell density to produce significant amounts of protein (see Garnier et al., Cytotechnol. 15:145-55, 1994). With either protocol, an expressed, secreted heterologous protein can be repeatedly isolated from the cell culture supernatant. Within the infected 293S cell production protocol, non-secreted proteins may also be effectively obtained.

ZTNFR-5 shares homology with OPG, a soluble TNF receptor involved in the regulation of bone density (Simonet et al., ibid.). Well established animal models are available to test the *in vivo* efficacy of ZTNFR-5 polypeptides for certain disease states, such as bone-related disorders. For example, the hypocalcemic rat model can be used to determine the effect of ZTNFR-5 on serum calcium, and the ovariectomized rat or mouse can be used as a model system for osteoporosis. Bone changes seen in these models and in humans during the early stages of estrogen deficiency are qualitatively similar.

ZTNFR-5 polypeptides can also be used to prepare antibodies that specifically bind to ZTNFR-5 epitopes, peptides or polypeptides. Methods for preparing polyclonal and monoclonal antibodies are well known in the art (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; and Hurrell, J. G. R., Ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, FL, 1982). As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, hamsters, guinea pigs and rats as well as transgenic animals such as transgenic sheep, cows, goats or pigs. Antibodies may also be expressed in yeast and fungi in modified forms as well as in mammalian and insect cells.

The ZTNFR-5 polypeptide or a fragment thereof serves as an antigen (immunogen) to inoculate an animal or elicit an immune response. Suitable antigens would include the ZTFNR-5 polypeptide encoded by SEQ ID NO:2 from amino acid residue 24-300 of SEQ ID NO:2, or a contiguous 9-300 amino acid residue fragment thereof. The immunogenicity of a ZTNFR-5 polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of ZTNFR-5 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments thereof, such as $F(ab')_2$ and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included. Non-human antibodies may be humanized by grafting only non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the

potential for adverse immune reactions upon administration to humans is reduced. Humanized monoclonal antibodies directed against ZTNFR-5 polypeptides could be used as a protein therapeutic, in particular for use as an immunotherapy. Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to ZTNFR-5 protein or peptide, and selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled ZTNFR-5 protein or peptide).

Antibodies are defined to be specifically binding if they bind to a ZTNFR-5 polypeptide with a binding affinity (K_a) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most preferably 10^9 M^{-1} or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art (for example, by Scatchard analysis).

A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to ZTNFR-5 proteins or peptides. Exemplary assays are described in detail in Antibodies: A Laboratory Manual, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition assay, and sandwich assay. In addition, antibodies can be screened for binding to wild-type versus mutant ZTNFR-5 protein or peptide.

Antibodies to ZTNFR-5 may be used for immunohistochemical tagging of cells that express human ZTNFR-5, for example, to use in a diagnostic assays; for isolating ZTNFR-5 by affinity purification; for screening expression libraries; for generating anti-idiotypic antibodies; and as neutralizing antibodies or as

antagonists to block ZTNFR-5 *in vitro* and *in vivo*. Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or labels may feature use of biotin-avidin or other complement/anti-complement pairs as intermediates. Antibodies herein may also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications.

Antibodies can be made to soluble, ZTNFR-5 polypeptides which are His or FLAG™ tagged. Alternatively, such polypeptides form a fusion protein with Human Ig. In particular, antiserum containing polypeptide antibodies to His-tagged, or FLAG™-tagged soluble ZTNFR-5 can be used in analysis of tissue distribution of ZTNFR-5 by immunohistochemistry on human or primate tissue. These soluble ZTNFR-5 polypeptides can also be used to immunize mice in order to produce monoclonal antibodies to a soluble human ZTNFR-5 polypeptide. Monoclonal antibodies to a soluble human ZTNFR-5 polypeptide can also be used to mimic ligand/receptor coupling, resulting in activation or inactivation of the ligand/receptor pair. For instance, it has been demonstrated that cross-linking anti-soluble CD40 monoclonal antibodies provides a stimulatory signal to B cells that have been sub-optimally activated with anti-IgM or LPS, and results in proliferation and immunoglobulin production. These same monoclonal antibodies act as antagonists when used in solution by blocking activation of the receptor. Monoclonal antibodies to ZTNFR-5 can be used to determine the distribution, regulation and biological interaction of the ZTNFR-5/ZTNFR-5-ligand pair on specific cell lineages identified by tissue distribution studies.

The invention also provides isolated and purified ZTNFR-5 polynucleotide probes or primers. Such polynucleotide probes can be RNA or DNA. DNA can be either cDNA or genomic DNA. Polynucleotide probes are single or double-stranded DNA or RNA, generally synthetic oligonucleotides, but may be generated from cloned cDNA or genomic sequences and will generally comprise at least 16 nucleotides, more often from 17 nucleotides to 25 or more nucleotides, sometimes 40 to 60 nucleotides, and in some instances a substantial portion, domain or even the entire ZTNFR-5 gene or cDNA. Probes and primers are generally synthetic oligonucleotides, but may be generated from cloned cDNA or genomic sequences or its complements. Analytical probes will generally be at least 20 nucleotides in length, although somewhat shorter probes (14-17 nucleotides) can be used. PCR primers are at least 5 nucleotides in length, preferably 15 or more nt, more preferably 20-30 nt. Short polynucleotides can be used when a small region of the gene is targeted for analysis. For gross analysis of genes, a polynucleotide probe may comprise an entire exon or more. Probes can be labeled to provide a detectable signal, such as with an enzyme, biotin, a radionuclide, fluorophore, chemiluminescer, paramagnetic particle and the like, which are commercially available from many sources, such as Molecular Probes, Inc., Eugene, OR, and Amersham Corp., Arlington Heights, IL, using techniques that are well known in the art. The synthetic oligonucleotides of the present invention have at least 80% identity to a representative ZTNFR-5 DNA sequence (SEQ ID NO:1) or its complements. Preferred regions from which to construct probes include the 5' and/or 3' coding sequences, ligand binding regions, and signal sequences, and the like. Techniques for developing polynucleotide probes and hybridization techniques are known in the art, see for example, Ausubel et al., eds.,

Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1991.

Such probes can also be used in hybridizations to detect the presence or quantify the amount of ZTNFR-5 gene or mRNA transcript in a sample. ZTNFR-5 polynucleotide probes could be used to hybridize to DNA or RNA targets for diagnostic purposes, using such techniques such as fluorescent *in situ* hybridization (FISH) or immunohistochemistry.

Polynucleotide probes can be used to identify genes encoding ZTNFR-5-like proteins. For example, ZTNFR-5 polynucleotides can be used as primers and/or templates in PCR reactions to identify other novel members of the TNFR family.

Such probes can also be used to screen libraries for related sequences encoding novel tumor necrosis factor receptors. Such screening would be carried out under conditions of low stringency which would allow identification of sequences which are substantially homologous, but not requiring complete homology to the probe sequence. Such methods and conditions are well known in the art, see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989. Such low stringency conditions could include hybridization temperatures less than 42°C, formamide concentrations of less than 50% and moderate to low concentrations of salt. Libraries may be made of genomic DNA or cDNA.

Polynucleotide probes are also useful for Southern, Northern, or slot blots, colony and plaque hybridization and *in situ* hybridization. Mixtures of different ZTNFR-5 polynucleotide probes can be prepared which would increase sensitivity or the detection of low copy number targets, in screening systems.

In addition, such polynucleotide probes could be used to hybridize to counterpart sequences on individual

chromosomes. Chromosomal identification and/or mapping of the ZTNFR-5 gene could provide useful information about gene function and disease association. Many mapping techniques are available to one skilled in the art, for example, mapping somatic cell hybrids, and fluorescence *in situ* hybridization (FISH). A preferred method is radiation hybrid mapping. Radiation hybrid mapping is a somatic cell genetic technique developed for constructing high-resolution, contiguous maps of mammalian chromosomes (Cox et al., <u>Science</u> <u>250</u>:245-50, 1990). Partial or full knowledge of a gene's sequence allows the designing of PCR primers suitable for use with chromosomal radiation hybrid mapping panels. Commercially available radiation hybrid mapping panels which cover the entire human genome, such as the Stanford G3 RH Panel and the GeneBridge 4 RH Panel (Research Genetics, Inc., Huntsville, AL), are available. These panels enable rapid, PCR based, chromosomal localizations and ordering of genes, sequence-tagged sites (STSs), and other non-polymorphic- and polymorphic markers within a region of interest. This includes establishing directly proportional physical distances between newly discovered genes of interest and previously mapped markers. The precise knowledge of a gene's position can be useful in a number of ways including: 1) determining if a sequence is part of an existing contig and obtaining additional surrounding genetic sequences in various forms such as YAC-, BAC- or cDNA clones, 2) providing a possible candidate gene for an inheritable disease which shows linkage to the same chromosomal region, and 3) for cross-referencing model organisms such as mouse which may be beneficial in helping to determine what function a particular gene might have.

Chromosomal localization can also be done using STSs. An STS is a DNA sequence that is unique in the human genome and can be used as a reference point for a particular chromosome or region of a chromosome. An STS

can be defined by a pair of oligonucleotide primers that can be used in a polymerase chain reaction to specifically detect this site in the presence of all other genomic sequences. Since STSs are based solely on DNA sequence they can be completely described within a database, for example, Database of Sequence Tagged Sites (dbSTS), GenBank, (National Center for Biological Information, National Institutes of Health, Bethesda, MD http://www.ncbi.nlm.nih.gov), they can be searched with a gene sequence of interest for the mapping data contained within these short genomic landmark STS sequences.

The present invention also provides reagents for use in diagnostic applications. For example, the ZTNFR-5 gene, a probe comprising ZTNFR-5 DNA or RNA, or a subsequence thereof can be used to determine if the ZTNFR-5 gene is present on a particular chromosome or if a mutation has occurred. Detectable chromosomal aberrations at the ZTNFR-5 gene locus include, but are not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. These aberrations can occur within the coding sequence, within introns, or within flanking sequences, including upstream promoter and regulatory regions, and may be manifested as physical alterations within a coding sequence or changes in gene expression level.

In general, these diagnostic methods comprise the steps of (a) obtaining a genetic sample from a patient; (b) incubating the genetic sample with a polynucleotide probe or primer as disclosed above, under conditions wherein the polynucleotide will hybridize to complementary polynucleotide sequence, to produce a first reaction product; and (iii) comparing the first reaction product to a control reaction product. A difference between the first reaction product and the control reaction product is indicative of a genetic abnormality in the patient. Genetic samples for use within the present

invention include genomic DNA, cDNA, and RNA. The polynucleotide probe or primer can be RNA or DNA, and will comprise a portion of SEQ ID NO:1, the complement of SEQ ID NO:1, or an RNA equivalent thereof. Suitable assay methods in this regard include molecular genetic techniques known to those in the art, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, ligation chain reaction (Barany, PCR Methods and Applications 1:5-16, 1991), ribonuclease protection assays, and other genetic linkage analysis techniques known in the art (Sambrook et al., ibid.; Ausubel et. al., ibid.; Marian, Chest 108:255-65, 1995). Ribonuclease protection assays (see, e.g., Ausubel et al., ibid., ch. 4) comprise the hybridization of an RNA probe to a patient RNA sample, after which the reaction product (RNA-RNA hybrid) is exposed to RNase. Hybridized regions of the RNA are protected from digestion. Within PCR assays, a patient's genetic sample is incubated with a pair of polynucleotide primers, and the region between the primers is amplified and recovered. Changes in size or amount of recovered product are indicative of mutations in the patient. Another PCR-based technique that can be employed is single strand conformational polymorphism (SSCP) analysis (Hayashi, PCR Methods and Applications 1:34-8, 1991).

Compounds identified as ZTNFR-5 agonists are useful for modifying the proliferation and development of target cells *in vitro* and *in vivo*. For example, agonist compounds are useful alone or in combination with other cytokines and hormones as components of defined cell culture media. Agonists are thus useful in specifically mediating the growth and/or development of ZTNFR-5-bearing T lymphocytes cells in culture. Agonists and antagonists may also prove useful in the study of effector functions of T lymphocytes, in particular T lymphocyte activation

and differentiation. Antagonists are useful as research reagents for characterizing ligand-receptor interaction.

Compounds identified as ZTNFR-5 agonists are useful for modifying the proliferation and development of target cells *in vitro* and *in vivo*. For example, agonist compounds are useful alone or in combination with other cytokines and hormones as components of defined cell culture media. Agonists are thus useful in specifically mediating the maturation of ZTNFR-5-bearing cells in culture.

The invention also provides antagonists, which either bind to ZTNFR-5 polypeptides or, alternatively, to a ligand to which ZTNFR-5 polypeptides bind, thereby inhibiting or eliminating the function of ZTNFR-5. Such ZTNFR-5 antagonists would include antibodies; oligonucleotides which bind either to the ZTNFR-5 polypeptide or to its ligand; natural or synthetic analogs of ZTNFR-5 ligands which retain the ability to bind the receptor but do not result in either ligand or receptor signaling. Such analogs could be peptides or peptide-like compounds. Natural or synthetic small molecules which bind to ZTNFR-5 polypeptides and prevent signaling are also contemplated as antagonists. As such, ZTNFR-5 antagonists would be useful as therapeutics for treating certain disorders where blocking signal from either a ZTNFR-5 receptor or ligand would be beneficial. Antagonists are useful as research reagents for characterizing ligand-receptor interaction.

ZTNFR-5 polypeptides may be used within diagnostic systems to detect the presence of ligand polypeptides. Antibodies or other agents that specifically bind to ZTNFR-5 may also be used to detect the presence of circulating receptor or ligand polypeptides. Such detection methods are well known in the art and include, for example, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay.

Immunohistochemically labeled ZTNFR-5 antibodies can be used to detect ZTNFR-5 receptor and/or ligands in tissue samples. ZTNFR-5 levels can also be monitored by such methods as RT-PCR, where ZTNFR-5 mRNA can be detected and quantified. The information derived from such detection methods would provide insight into the significance of ZTNFR-5 polypeptides in various diseases, and as a would serve as diagnostic tools for diseases for which altered levels of ZTNFR-5 are significant. Altered levels of ZTNFR-5 receptor polypeptides may be indicative of pathological conditions including cancer, autoimmune disorders, bone disorders, inflammation and immunodeficiencies.

The ZTNFR-5 polynucleotides and/or polypeptides, agonists and antagonists disclosed herein can be useful as therapeutics to modulate one or more biological processes in cells, tissues and/or biological fluids.

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1
Identification of ZTNFR-5

Novel ZTNFR-5 encoding polynucleotides and polypeptides of the present invention were initially identified by querying an EST database for sequences homologous to conserved motifs within the TNF ligand family. Using this information, 16 independent ESTs were identified and aligned to produce a novel human contig of about 1181 bp. To identify the corresponding cDNA, a clone considered likely to contain the entire sequence was used for sequencing. Using a QIAwell 8 plasmid kit (Qiagen, Inc., Chatsworth, CA) according to manufacturer's instructions, a 5 ml overnight culture in LB + 50 µg/ml ampicillin was prepared. The template was sequenced on an

Applied Biosystems™ model 377 DNA sequencer (Perkin-Elmer Cetus, Norwalk, Ct.) using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corp.) according to the manufacturer's instructions. Oligonucleotides ZC694 (SEQ ID NO:4) and ZC695 (SEQ ID NO:5) to the T7 and SP6 promoters on the vector were used as sequencing primers. Oligonucleotides ZC14153 (SEQ ID NO:6), ZC14343 (SEQ ID NO:7), ZC14344 (SEQ ID NO:8), ZC14331 (SEQ ID NO:9) and ZC14467 (SEQ ID NO:10) were used to complete the sequence from the clone. Sequencing reactions were carried out in a Hybaid OmniGene Temperature Cycling System (National Labnet Co., Woodbridge, NY). SequencherTM 3.0 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI) was used for data analysis. The resulting 1205 bp sequence is disclosed in SEQ ID NO:1.

Example 2
Tissue Distribution

Human Multiple Tissue Northern Blots (MTN I, MTN II, and MTN III; Clontech) were probed to determine the tissue distribution of human ZTNFR-5 expression. An approximately 397 bp (SEQ ID NO:11) PCR derived probe to a region near the predicted N-terminus of the deduced amino acid sequence of the of the contig was amplified from a pool of human heart, uterus, K565, αMCf7 derived Marathon™-ready cDNA libraries. Oligonucleotide primers ZC13,780 (SEQ ID NO:12) and ZC13,793 (SEQ ID NO:13) were designed based on a less than full length contig assembly. The Marathon™-ready cDNA libraries were prepared according to manufacturer's instructions (Marathon™ cDNA Amplification Kit; Clontech, Palo Alto, CA) using human heart, uterus, K562 and αMCF7 poly A+ RNA (Clontech). The probe was amplified in a polymerase chain reaction as follows: 1 cycle at 94°C for 1 minute 30 seconds; 35 cycles of 94°C for 10 seconds, 62°C for 20 seconds, and 72°C for

30 seconds, followed by 1 cycle at 72°C for 10 minutes. The resulting DNA fragment was electrophoresed on a 2% low melt agarose gel (SEA PLAQUE GTG low melt agarose, FMC Corp., Rockland, ME), the fragment was purified using the QIAquick™ method (Qiagen, Chatsworth, CA), and the sequence was confirmed by sequence analysis. The probe was radioactively labeled using the random priming MULTIPRIME DNA labeling system (Amersham, Arlington Heights, IL), according to the manufacturer's specifications. The probe was purified using a NUCTRAP push column (Stratagene, La Jolla, CA). ExpressHyb™ (Clontech) solution was used for prehybridization and as a hybridizing solution for the Northern blots. Hybridization took place overnight at 55°C using 2.9 x 10^6 cpm/ml of labeled probe. The blots were then washed at 65°C in 0.1X SSC, 0.1% SDS. Signal intensity was highest in lung, spinal cord, stomach, lymph node, spleen, colon and trachea. Less intense signals were present in kidney, thymus, heart, pancreas, prostate, small intestine, placenta, thyroid, and bone marrow tissue. The transcript size was approximately 1.2 kb. In the case of lung a strongly hybridizing 2.4 kb transcript was also seen.

Example 3
Preparation of ZTNFR-5-Ig Fusion Vectors

Preparation of mammalian and BV expression vectors containing soluble mutated IgFc

To prepare the ZTNFR-5-Ig fusion protein the Fc region of human IgG1 (the hinge region and the CH2 and CH3 domains) was modified. The Fc region was isolated from a human fetal liver library (Clontech) by PCR using oligo primers ZC10,134 (SEQ ID NO:15) and ZC10,135 (SEQ ID NO:16).

PCR was used to introduce mutations within the Fc region to reduce FcgRI binding. The FcgRI binding site (Leu-Leu-gly-Gly) was mutated to Ala-Glu-gly-Ala (amino

acid residues 38-41 of SEQ ID NO:17) according to Baum et al. (EMBO J. 13:3992-4001, 1994), to reduce FcR1 binding (Duncan et al., Nature 332:563-4, 1988). Oligonucleotide primers ZC15,345 (SEQ ID NO:18) and ZC15,347 (SEQ ID NO:19) were used to introduce the mutation. To a 50 µl final volume was added 570 ng IgFc template, 5 µl 10X Pfu Reaction Buffer (Stratagene), 8 µl of 1.25 mM dNTPs, 31 µl dH_2O 2 µl of 20 mM ZC15,345 (SEQ ID NO:18), and 2 µl 20 mM ZC15,347 (SEQ ID NO:19). An equal volume of mineral oil was added and the reaction was heated to 94°C for 1 minute. Pfu polymerase (2.5 units, Stratagene) was added followed by 25 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute followed by a 7 minute extension at 72°C. The reaction products were electrophoresed and the band corresponding to the predicted size of ~676 bp was detected. The band was excised from the gel and recovered using a QIAGEN QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturers instructions.

PCR was also used to introduce a mutation of Ala to Ser (amino acid residue 134 of SEQ ID NO:17) and Pro to Ser (amino acid residue 135 of SEQ ID NO:17) to reduce complement C1q binding and/or complement fixation (Duncan and Winter, Nature 332:788, 1988) and add a 5' Bam HI restriction site, a signal sequence for secretion, a 3' Xba I restriction site as well as the stop codon TAA. Two first round reactions were done using the FcgRI binding site mutated IgFc sequence as a template. To a 50 µl final volume was added 1 µl FcgRI binding site mutated IgFc template, 5 µl 10X Pfu Reaction Buffer (Stratagene), 8 µl 1.25 mM dNTPs, 31 µl dH_2O, 2 µl 20 mM ZC15,517 (SEQ ID NO:20), a 5' primer beginning at nucleotide 36 of SEQ ID NO:17 and 2 µl 20 mM ZC15,530 (SEQ ID NO:21), a 3' primer beginning at the complement of nucleotide 405 of SEQ ID NO:17. The second reaction contained 2 µl of 20 mM

each of oligonucleotide primers ZC15,518 (SEQ ID NO:22), a 5' primer beginning at nucleotide 388 of SEQ ID NO:17 and ZC15,347 (SEQ ID NO:19), a 3' primer, to introduce the Ala to Ser mutation, Xba I restriction site and stop codon. An equal volume of mineral oil was added and the reactions were heated to 94°C for 1 minute. Pfu polymerase (2.5 units, Stratagene) was added followed by 25 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 2 minutes followed by a 7 minute extension at 72°C. The reaction products were electrophoresed and bands corresponding to the predicted sizes, ~370 and ~395 bp respectfully, were detected. The bands were excised from the gel and extracted using a QIAGEN QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturers instructions. A second round reaction was done to join the above fragments and add the 5' Bam HI restriction site. To a 50 µl final volume was added 30 µl dH_2O, 8 µl 1.25 mM dNTPs, 5 µl 10X Pfu polymerase reaction buffer (Stratagene) and 1 µl each of the two first round PCR products. An equal volume of mineral oil was added and the reaction was heated to 94°C for 1 minute. Pfu polymerase (2.5 units, Stratagene) was added followed by 5 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 2 minutes. The temperature was again brought to 94°C and 2 µl each of 20 mM ZC15,516 (SEQ ID NO:23), a 5' primer beginning at nucleotide 1 of SEQ ID NO:17, introducing a Bam HI restriction site, and 20 mM ZC15,347 (SEQ ID NO:19) were added followed by 25 cycles at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes, and a final 7 minute extension at 72°C. A portion of the reaction was visualized using gel electrophoresis. A 789 bp band corresponding the predicted size was detected. The remainder of the IgFc PCR fragment and the baculovirus expression vector pFBL2 (pFASTBacTM (Gibco BRL) with baculo basic protein promoter added in place of existing promoter) were digested with the restriction enzymes Bam HI and Xba I. The IgFc

fragment was ligated into the vector at a molar ratio of insert to vector of approximately 4 to 1. The IgFc containing pFBL2 vector was then used to transform competent *E. coli* DH10B cells (Life Technologies). The transformation reaction consisted of 1 µl of the ligation reaction in 100 µl competent cells. The transformation reaction was incubated for 30 minutes on ice, heat shocked at 42°C for 40 seconds, and incubated on ice for 2 minutes. One half microliter of Luria Broth (LB) was added to the transformation reaction and it was then plated on LB plus ampicillin (100 mg/ml) plates and incubated overnight as 37°C. To screen for positive transformants, 5 colonies were cultured overnight in LB containing 100 mg/ml ampicillin. DNA was prepared from each culture using a QIAGEN QIAprep Spin Miniprep Kit (QIAGEN) according the manufacturer's instruction. Restriction digests were done to determine proper insertion of the fragment. The mutated IgFc sequence (SEQ ID NO:17) of a positive clone, designated IgFc4/pFBL2, was verified by sequence analysis.

A second expression vector, mammalian vector pHZ200, was digested with restriction enzymes Bam HI and Xba I and ligated to the IgFc fragment as described above. pHZ200 is an expression vector that may be used to express protein in mammalian cells or in a frog oocyte translation system from mRNAs that have been transcribed *in vitro*. The pHZ200 expression unit comprises the mouse metallothionein-1 promoter, the bacteriophage T7 promoter flanked by multiple cloning banks containing unique restriction sites for insertion of coding sequences, the human growth hormone terminator and the bacteriophage T7 terminator. In addition, pHZ200 contains an *E. coli* origin of replication; a bacterial beta lactamase gene; a mammalian selectable marker expression unit comprising the SV40 promoter and origin, a dihydrofolate reductase gene and the SV40 transcription terminator. Colonies were

screened by PCR using primers, ZC6583 (SEQ ID NO:24), a sequence from the pHZ200 expression vector, and ZC15,530 (SEQ ID NO:21). A sample of each colony in 5 µl LB was added to 27 µl dH_2O, 5 µl 10X Advantage cDNA Polymerase Mix Buffer, 8 µl 1.25 mM dNTP, 2 µl each 20 mM ZC6583 (SEQ ID NO:24) and ZC15,530 (SEQ ID NO:21), and 1 µl Advantage cDNA Polymerase Mix (Clontech). The reactions were heated at 94°C for 1 minute followed by 25 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 2 minutes followed by a 7 minute extension at 68°C. Positive clones were identified by the presence of a band at 486 bp. The insert sequence of a positive clone, designated IgFc4/pHZ200, was verified by sequence analysis.

Preparation of mammalian and BV expression vectors containing ZTNFR5-mutated IgFc fusion gene

A soluble ZTNFR-5 fragment was prepared using PCR, truncating the transmembrane and cytoplasmic domains. Oligonucleotide primer ZC15,334 (SEQ ID NO:25) adds a Bam HI restriction site to the 5' side of nucleotide 181 in SEQ ID NO:1 and oligonucleotide primer ZC15,519 (SEQ ID NO:26) adds sequence encoding for amino acid residues Glu and Pro and a Bgl II site just 3' to nucleotide 1892 in SEQ ID NO:1. Between 10 and 100 ng of plasmid DNA, described in Example 1, was added to 32 µl dH_2O, 5 µl 10X Pfu Reaction Buffer (Stratagene), 8 µl of 1.25 mM dNTPs, 1 ml of 40 mM ZC15,334 (SEQ ID NO:25), and 2 µl 20 mM ZC15,519 (SEQ ID NO:26). The reaction was heated to 94°C for 1 minute. Pfu polymerase (2.5 units, Stratagene) was added followed by 25 cycles at 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 2 minutes followed by a 7 minute extension at 72°C. The reaction products were electrophoresed and the band corresponding to the predicted size of ~926 bp was detected. The band was excised from the gel and recovered using a QIAGEN

QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturers instructions. The soluble ZTNFR5 PCR fragment was ligated in pCR®-Blunt (Clontech) following the manufacturer's recommended protocol for ligation and transformation of *E. coli* TOP10 competent cells. Positive clones were selected by PCR screening using pCR®-Blunt oligonucleotide primers ZC447 (SEQ ID NO:27) and ZC694 (SEQ ID NO:4). A sample of each colony in 5 ml LB was added to 27 µl dH_2O, 5 µl 10X Advantage cDNA Polymerase Mix Buffer, 8 µl 1.25 mM dNTP, 2 µl each 20 mM ZC447 (SEQ ID NO:27) and ZC694 (SEQ ID NO:4), and 1 µl Advantage cDNA Polymerase Mix (Clontech). The reactions were heated at 94°C for 1 minute followed by 25 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 2 minutes followed by a 7 minute extension at 68°C. Positive clones were identified by the presence of a band at ~1153 bp. The insert sequence of a positive clone, designated ztnfr5/pCR-Blunt, was verified by sequence analysis.

Ztnfr5/pCR-Blunt was digested with restriction enzymes Bam HI and Bgl II to release a fragment containing the ztnfr5 signal sequence and extracellular domain fragment. The fragment was isolated by gel electrophoresis as described above and ligated into a Bam HI and Bgl II digested IgFc4/pHZ200 vector as described above at a 4 to 1 molar ratio of insert to vector. Competent DH10B cells were transformed with the ligation product, plated and a clone containing the correct insert was identified by restriction enzyme mapping analysis and designated ZTNFR5Fc4/pHZ200. In a second ligation, the Bam HI-Xba I fragment containing the ZTNFR5-IgFc fusion protein coding region from ZTNFR5Fc4/pHZ200 was ligated into Bam HI-Xba I digested pFBL2 vector as described above at a 4 to 1 molar ration of insert to vector. *E. coli* DH10B competent cells (Life Technologies) were transformed with the ligation product according to manufacturer's instructions and a clone containing the correct insert was

identified by restriction enzyme mapping analysis and designated ZTNFR5Fc4/pFBL2.

Example 5
Mammalian Expression of Soluble ZTNFR5

BHK 570 cells (ATCC NO: CRL-10314) were plated in 10 cm tissue culture dishes and allowed to grow to approximately 50 to 70% confluency overnight at 37°C, 5% CO_2, in DMEM/FBS media (DMEM, Gibco/BRL High Glucose, (Gibco BRL, Gaithersburg, MD), 5% fetal bovine serum (Hyclone, Logan, UT), 1 μM L-glutamine (JRH Biosciences, Lenexa, KS), 1 μM sodium pyruvate (Gibco BRL)). The cells were then transfected with the plasmid ztnfr5Fc4/pHZ200, using Lipofectamine™ (Gibco BRL), in serum free (SF) media formulation (DMEM, 10 mg/ml transferrin, 5 mg/ml insulin, 2 mg/ml fetuin, 1% L-glutamine and 1% sodium pyruvate). Sixteen micrograms of ztnfr5Fc4/pHZ200 was diluted into a 15 ml tube to a total final volume of 640 μl with SF media. In a separate tube, 35 μl of Lipofectamine™ (Gibco BRL) was mixed with 605 μl of SF medium. The Lipofectamine™ mix was added to the DNA mix and allowed to incubate approximately 30 minutes at room temperature. Five milliliters of SF media was added to the DNA:Lipofectamine™ mixture. A chosen 10 cm plate of cells was rinsed once with 5 ml of SF media, aspirated, and the DNA:Lipofectamine™ mixture was added dropwise. The cells were incubated at 37°C for five hours, then 6.4 ml of DMEM/10% FBS, 1% PSN media was added to each plate. The plates were incubated at 37°C overnight and the DNA:Lipofectamine™ mixture was replaced with fresh FBS/DMEM media the next day. On day 2 post-transfection, the cells were split into the selection media (DMEM/FBS media from above with the addition of 1 μM methotrexate (Sigma Chemical Co., St. Louis, Mo.)) in 150 mm plates at 1:50, 1:100 and 1:200. The plates were refed at day 5 post-transfection with fresh selection media.

Screening colonies

Approximately 10-12 days post-transfection, one 150 mm culture dish of methotrexate resistant colonies was chosen, the media aspirated, the plates washed with 10 ml serum-free ESTEP 2 media (668.7g/50L DMEM (Gibco), 5.5 g/50L pyruvic acid, sodium salt 96% (Mallinckrodt), 185.0 g/50L $NaHCO_3$ (Mallinkrodt), 5.0 mg/ml, 25 ml/50L insulin, 10.0 mg/ml and 25 ml/50 L transferrin). The wash media was aspirated and replaced with 5 ml serum-free ESTEP 2. A sterile Teflon mesh (Spectrum Medical Industries, Los Angeles, CA) pre-soaked in serum-free ESTEP 2 was then placed over the cells. A sterile nitrocellulose filter pre-soaked in serum-free ESTEP 2 was then placed over the mesh. Orientation marks on the nitrocellulose were transferred to the culture dish. The plates were then incubated for 5 hours in a 37°C, 5% CO_2 incubator. Following incubation, the filter was removed, and the media aspirated and replaced with DMEM/5% FBS, 1X PSN (Gibco BRL) media. The filters were blocked in 2.5% nonfat dry milk/Western A buffer (Western A: 50mM Tris pH 7.4, 5 mM EDTA, 0.05% NP-40, 150 mM NaCl and 0.25% gelatin) overnight at 4°C. The filter was then incubated with a goat anti-human IgG-HRP antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) at a 1:2000 dilution in 2.5% nonfat dry milk/Western A buffer (Western A: 50mM Tris pH 7.4, 5 mM EDTA, 0.05% NP-40, 150 mM NaCl and 0.25% gelatin) for 1 hour at room temperature on a rotating shaker. The filter was then washed three times at room temperature in PBS plus 0.1% Tween 20, 5-15 minutes per wash. The filter was developed with ECL reagent (Amersham Corp., Arlington Heights, IL) according the manufacturer's directions and exposed to film (Hyperfilm ECL, Amersham) for approximately 1 second.

The film was aligned with the plate containing the colonies. Using the film as a guide, 12 suitable colonies were selected. Sterile, 3 mm coloning discs (PGC

Scientific Corp., Frederick, MD) were soaked in trypsin, and placed on the colonies. Twelve colonies were transferred into 200 μl of selection medium in a 96 well plate. A series of seven, two-fold dilutions were carried out for each colony. The cells were grown for one week at 37°C at which time the wells which received the lowest dilution of cells which are now at the optimum density were selected, trypsinized and transferred to a 12 well plate containing selection media. When confluent the cells were transferred into 2 T-75 flasks, one flask switched to serum free media, harvested and subjected to Western analysis.

Example 6
Baculovirus Expression of Soluble ZTNFR5

One microliter of the ztnfr5Fc4/pFBL2 construct described above was used to transform 20 µl DH10Bac Max Efficiency competent cells (GIBCO-BRL, Gaithersburg, MD) according to manufacturer's instruction, by heat shock at 42°C for 45 seconds. The transformants were then diluted in 980 µl SOC media and plated on to Luria Agar plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, IPTG and Bluo Gal. The cells were incubated for 48 hours at 37°C. A color selection was used to identify those cells having virus that had incorporated into the plasmid (referred to as a "bacmid"). Those colonies, which were white in color, were picked for analysis. Bacmid DNA was isolated from positive colonies and used to transfect *Spodoptera frugiperda* (Sf9) cells.

Sf9 cells were seeded at 5 x 10^6 cells per 35 mm plate and allowed to attach for 1 hour at 27°C. Five microliters of bacmid DNA was diluted with 100 µl Sf-900 II SFM. Six microliters of CellFECTIN Reagent (Life Technologies) was diluted with 100 µl Sf-900 II SMF. The bacmid DNA and lipid solutions were gently mixed and incubated 30-45 minutes at room temperature. The media the plate of cells was aspirated, and the lipid-DNA mixture to which 0.8 ml of Sf-900 II SFM was added. The cells were incubated at 27°C for 4 hours, then 2 ml of Sf-900 II media containing penicillin/streptomycin was added to each plate. The plates were incubated at 27°C, 90% humidity, for 48 hours after which the virus was harvested.

Primary Amplification

Sf9 cells were grown in 50 ml Sf-900 II SFM in a shake flask to an approximate density of 0.50 x 10^6 cells/ml. They were then transfected with 50 µl of the virus stock from above and incubated at 27°C for 2 days

after which time the virus was harvested, titer 2.2×10^7 pfu/ml. To scale up, Sf9 cells were grown to a density of 1.7×10^6 SF9 cells/ml in 5 liter batches. The cells were then transfected with the harvested virus (MOI 3) and incubated as above for 46 hours followed by harvest.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim:

1. An isolated polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2.

2. An isolated polypeptide according to claim 1, wherein said polypeptide comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 300, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2.

3. An isolated polypeptide according to claim 2, wherein said polypeptide comprises the region between amino acid residue 1 and amino acid residue 300 of SEQ ID NO:2.

4. An isolated polypeptide according to claim 2, further comprises an affinity tag.

5. A fusion protein consisting essentially of a first portion and a second portion joined by a peptide bond, said first portion comprising a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70,

73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2; and

said second portion comprising another polypeptide.

6. A fusion protein according to claim 5, wherein said second portion is an IgG Fc region.

7. A fusion protein comprising a secretory signal sequence having the amino acid sequence of amino acid residues 1-23 of SEQ ID NO:2, wherein said secretory signal sequence is operably linked to an additional polypeptide.

8. A pharmaceutical composition comprising an isolated polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2; in combination with a pharmaceutically acceptable vehicle.

9. An isolated polynucleotide encoding an isolated polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2.

10. An isolated polynucleotide encoding a polypeptide according to claim 9, wherein said polypeptide comprises an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 300, wherein said

polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2.

11. An isolated polypeptide according to claim 10, wherein said polypeptide comprises the region between amino acid residue 1 and amino acid residue 300 of SEQ ID NO:2.

12. An isolated polynucleotide encoding a polypeptide according to claim 10, further comprising an affinity tag.

13. An isolated polynucleotide according to claim 11, selected from the group consisting of,

a) a polynucleotide having a sequence of nucleotides from nucleotide 252 to nucleotide 764 of SEQ ID NO:1;

b) a polynucleotide having a sequence of nucleotides from nucleotide 252 to nucleotide 1082 of SEQ ID NO:1;

c) a polynucleotide having a sequence of nucleotides from nucleotide 183 to nucleotide 764 of SEQ ID NO:1;

d) a polynucleotide having a sequence of nucleotides from nucleotide 183 to nucleotide 1082 of SEQ ID NO:1;

e) a polynucleotide having a sequence of nucleotides from nucleotide 1 to nucleotide 1205 of SEQ ID NO:1;

f) nucleotide sequences complementary to a), b), c), d) or e) and

g) degenerate nucleotide sequences of a), b), c), d), e) or f).

14. An expression vector comprising the following operably linked elements:

a transcription promoter;

a DNA segment encoding a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2; and

a transcription terminator.

15. An expression vector according to claim 14, wherein said DNA segment encodes a polypeptide comprising an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 300, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2.

16. An expression vector according to claim 15, wherein said DNA segment encodes a polypeptide comprising residues 24-194 of SEQ ID NO:2.

17. An expression vector according to claim 15, wherein said DNA segment encodes a polypeptide covalently linked amino terminally or carboxy terminally to an affinity tag.

18. An expression vector according the claim 17, wherein said secretory signal sequence comprises residues 1-23 of SEQ ID NO:2 or SEQ ID NO:44.

19. A cultured cell into which has been introduced an expression vector comprising the following operably linked elements:

a transcription promoter;

a DNA segment encoding a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2; and

a transcription terminator, wherein said cell expresses said polypeptide encoded by said DNA segment.

20. A method of producing a polypeptide comprising:

culturing a cell into which has been introduced an expression vector comprising the following operably linked elements:

a transcription promoter;

a DNA segment encoding a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2; and

a transcription terminator;

whereby said cell expresses said polypeptide encoded by said DNA segment; and

recovering said expressed polypeptide.

21. An antibody that specifically binds to an epitope of a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88,

91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2.

22. A binding protein that specifically binds to an epitope of a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:2 from amino acid residue 24 to amino acid residue 194, wherein said polypeptide has four extracellular, cysteine-rich pseudo-repeats having cysteine residues corresponding to amino acid residues 49, 52, 62, 70, 73, 88, 91, 95, 105, 113, 115, 126, 132, 150, 153, 168, 174 and 193 of SEQ ID NO:2.

23. An isolated polynucleotide comprising the sequence of nucleotide 1 to nucleotide 900 of SEQ ID NO:14.

24. An oligonucleotide probe or primer comprising at least 14 contiguous nucleotides of a polynucleotide of SEQ ID NO:14 or a sequence complementary to SEQ ID NO:14.

```
                  ztnfr5 predicted cleavage vv
                             .         .         .         .         .
ztnfr5            1 MRALEGPGLSLLCLVLALPALLPVPAVRGVAETPTYPWRDAETGERLVCA 50
                             |||  |     : :             | |   | ||   .|.|
OPG               1 .......MNKLLCCALVF.LDISIKWTTQETFPPKYLHYDEETSHQLLCD 42
                          OPG predicted cleavage^^                    |-

                             .         .         .         .         .
ztnfr5           51 QCPPGTFVQRPCRRDSPTTCGPCPPRHYTQFWNYLERCRYCNVLCGEREE 100
                    .|||||:... |       | | |||  :||   |.  : | ||. .| | :
OPG              43 KCPPGTYLKQHCTAKWKTVCAPCPDHYYTDSWHTSDECLYCSPVCKELQY 92
                    - cys-rich rep #1 --||----- cys-rich repeat #2 ---

                             .         .         .         .         .
ztnfr5          101 EARACHATHNRACRCRTGFFAHAGFCLEHASCPPGAGVIAPGTPSQNTQC 150
                      . |. |||| | |: | :    |||.| ||||| ||:  ||| .|| |
OPG              93 VKQECNRTHNRVCECKEGRYLEIEFCLKHRSCPPGFGVVQAGTPERNTVC 142
                    ------------||---- cys-rich repeat #3 ------------

                             .         .         .         .         .
ztnfr5          151 QPCPPGTFSASSSSSEQCQPHRNCTALGLALNVPGSSSHDTLCTSCTGFP 200
                    . || | ||  .||    |. | ||.  || |   |...|| :|.  .
OPG             143 KRCPDGFFSNETSSKAPCRKHTNCSVFGLLLTQKGNATHDNICSGNSEST 192
                    ||---------- cys-rich repeat #4 -----------|
```

FIG. 1A

```
                     .         .         .         .         .
ztnfr5  201 LSTRVPGAEECERAVIDFVAFQDISIKRLQRLLQALEAPE.GWGPTPRAG 249
                :     || |   |      .   |  |.  |   .       |
OPG     193 QKCGI.DVTLCEEAFFRFAVPTKFTPNWLSVLVDNLPGTKVNAESVERIK 241

                     .         .         .         .         .
ztnfr5  250 RAALQLKLRRRLTELLGAQ..DGALLVRLLQALRVARMPGLERSVRERFL 297
            |     .   .| .|   |  |  :. :::| : .     .:| :    |
OPG     242 RQHSSQEQTFQLLKLWKHQNKDQDIVKKIIQDIDLCE.NSVQRHIGHANL 290

                     .         .         .         .         .
ztnfr5  298 PVH............................................... 300

OPG     291 TFEQLRSLMESLPGKKVGAEDIEKTIKACKPSDQILKLLSLWRIKNGDQD 340

                     .         .         .         .         .
ztnfr5  300 .................................................. 300

OPG     341 TLKGLMHALKHSKTYHFPTNCHSESKEDHQVPSQLHNVQIVSEVIFRNDR 390
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FIG. 1B

SEQUENCE LISTING

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Arg Asp Ser Pro Thr Thr Cys Gly Pro Cys Pro Pro Arg His Tyr Thr
65 70 75

cag ttc tgg aac tac ctg gag cgc tgc cgc tac tgc aac gtc ctc tgc 467
Gln Phe Trp Asn Tyr Leu Glu Arg Cys Arg Tyr Cys Asn Val Leu Cys
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Ser Ser Ser Ser Ser Glu Gln Cys Gln Pro His Arg Asn Cys Thr Ala
165 170 175
Leu Gly Leu Ala Leu Asn Val Pro Gly Ser Ser Ser His Asp Thr Leu
180 185 190
Cys Thr Ser Cys Thr Gly Phe Pro Leu Ser Thr Arg Val Pro Gly Ala
195 200 205
Glu Glu Cys Glu Arg Ala Val Ile Asp Phe Val Ala Phe Gln Asp Ile
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Cys Pro Asp His Tyr Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys
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Leu Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu
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Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg
    130                 135                 140
Cys Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys
145                 150                 155                 160
Arg Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys
                165                 170                 175
Gly Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr
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305                 310                 315                 320
Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn
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Gly Asp Gln Asp Thr Leu Lys Gly Leu Met His Ala Leu Lys His Ser
            340                 345                 350
Lys Thr Tyr His Phe Pro Thr Asn Cys His Ser Glu Ser Lys Glu Asp
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agggctgggg tccgacacca agggcgggcc gcgcggcctt gcagctgaag ctgcgtcggc 960
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        1               5                  10
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aga tgg gtc ctg tcc gag ccc aga tct tca gac aaa act cac aca tgc 96
Arg Trp Val Leu Ser Glu Pro Arg Ser Ser Asp Lys Thr His Thr Cys
15 20 25 30

cca ccg tgc cca gca cct gaa gcc gag ggg gca ccg tca gtc ttc ctc 144
Pro Pro Cys Pro Ala Pro Glu Ala Glu Gly Ala Pro Ser Val Phe Leu
35 40 45

ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag 192
Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu
50 55 60

gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag 240
Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys
65 70 75

ttc aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag 288
Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys
80 85 90

ccg cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc 336
Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu
95 100 105 110

acc gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag 384
Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys
115 120 125

gtc tcc aac aaa gcc ctc cca tcc tcc atc gag aaa acc atc tcc aaa 432
Val Ser Asn Lys Ala Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys
130 135 140

gcc aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc 480
Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser
145 150 155

cgg gat gag ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa 528
Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys
160 165 170

ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag 576
Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln
175 180 185 190

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Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
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tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag      672
Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
            210                 215                 220

cag ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac      720
Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn
        225                 230                 235

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<400> 21
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<211> 42
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<223> Oligonucleotide ZC6583

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<210> 25
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<211> 38
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<220>
<223> Oligonucleotide ZC15519

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<220>
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<400> 27
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<210> 28
<211> 41
<212> PRT
<213> Artificial Sequence

<220>
<223> Tumor necrosis factor receptor pseudo-repeat #1

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<223> Tumor necrosis factor receptor pseudo-repeat #2

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<223> Xaa is any amino acid residue

<400> 29
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<212> PRT
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            20                  25                  30
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<220>

<223> Tumor necrosis factor receptor pseudo-repeat #3
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<400> 31

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            20                  25                  30
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa
        35                  40                  45
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<210> 32
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<223> Tumor necrosis factor receptor pseudo-repeat #4 motif

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or not present

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<400> 32

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Cys Xaa Xaa Cys Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
            20                  25                  30
Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa
        35                  40
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<210> 33
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<212> PRT
<213> Artificial Sequence

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<223> Tumor necrosis factor receptor pseudo-repeat #4
second motif

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or not present

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<223> Each Xaa is independently any amino acid residue
or not present

<221> VARIANT
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<223> Xaa is any amino acid residue

<400> 33

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Cys Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
20 25 30

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa
35 40

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/15072

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/62 C12N15/86 C12N5/10 C07K14/705
C07K14/735 C07K16/28 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 30694 A (HUMAN GENOME SCIENCES INC ;FENG PING (US); NI JIAN (US); EBNER REI) 16 July 1998 see abstract see example 3 seq. IDs 1,2 see claims 1-23 ---	1-5,8-24
E	EP 0 861 850 A (SMITHKLINE BEECHAM CORP) 2 September 1998 seq. IDs 1,2 see abstract ---	1-3, 9-11, 13-16, 19-22

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 1998	08/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 23614 A (AMGEN INC ;LACEY DAVID L (US); BOYLE WILLIAM J (US); CALZONE FRANK) 3 July 1997 see abstract see examples 7,8 see claims 1-60	1-24
A	US 5 447 851 A (BEUTLER BRUCE A ET AL) 5 September 1995 see abstract	6
A	DATABASE GENBANK Accession No. AA025673, 16 August 1996 HILLIER ET AL.: "H. sapiens cDNA clone IMAGE 366305" XP002085906 see abstract	1-24

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/15072

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			AU	6238698	A	03-08-1998
			WO	9830693	A	16-07-1998
EP 0861850	A	02-09-1998	CA	2220852	A	03-08-1998
			JP	10215886	A	18-08-1998
WO 9723614	A	03-07-1997	AU	1468697	A	17-07-1997
			CA	2210467	A	03-07-1997
			CN	1182452	A	20-05-1998
			DE	19654610	A	26-06-1997
			EP	0784093	A	16-07-1997
			EP	0870023	A	14-10-1998
			FR	2742767	A	27-06-1997
			GB	2312899	A	12-11-1997
			HU	9801122	A	28-08-1998
			NO	973699	A	21-10-1997
			PL	321938	A	05-01-1998
US 5447851	A	05-09-1995	NONE			